

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
15 July 2004 (15.07.2004)

PCT

(10) International Publication Number
WO 2004/059282 A2

- (51) International Patent Classification⁷: **G01N** (74) Agent: MADSEN, Matthew, L.; Howrey Simon Arnold & White LLP, 750 Bering Drive, Houston, TX 77057-2198 (US).
- (21) International Application Number: PCT/US2003/040192
- (22) International Filing Date:
17 December 2003 (17.12.2003)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/435,540 19 December 2002 (19.12.2002) US
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- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (regional): ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: METHOD AND MEANS FOR EARLY DETECTION OF PREGNANCY IN ANIMALS BY COMBINATION TESTING

	ISG 17	Progesterone	ISG 17	Progesterone	Cow
Test			+	+	Pregnant
Control					
Test			+	-	Open
Control					
Test			-	+	Open
Control					
Test			-	-	Open
Control					

(57) Abstract: Disclosed are methods for determining the pregnancy status of an animal, suspected of being pregnant, using combination tests comprising the analysis of progesterone and ubiquitin cross-reactive protein (UCRP), which is also known as interferon-stimulated gene product (ISG). The present invention provides for methods of diagnosing pregnancy at very early stages. The benefit of this early diagnosis is that by identifying animals which are not pregnant very shortly after breeding, allows for timely re-breeding thereby minimizing the amount of time the animal is open. Also disclosed are methods for making a breeding decision for an animal. These methods allow a herd manager to make breeding decisions based on the levels of UCRP and progesterone detected in a biological sample take from an animal suspected of being in the early stages of pregnancy.

WO 2004/059282 A2



Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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METHOD AND MEANS FOR EARLY DETECTION OF PREGNANCY IN ANIMALS BY COMBINATION TESTING

This application claims the benefit of United States provisional application serial number
5 60/435,540, filed December 19, 2002.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the fields of veterinary medicine, reproductive
10 biology and diagnostics. More specifically, the present invention relates to improved methods
for early stage pregnancy detection in animals, particularly in bovines and other ruminants.

2. Description of Related Art

Pregnancy diagnosis is an important component in sound reproductive management,
15 particularly in the dairy industry (Oltenacu *et al.*, 1990), where a high proportion of artificial
inseminations fail (Streenan and Diskin, 1986). A reliable yet simple pregnancy test for cattle
has long been sought. Several procedures are available, including a milk progesterone assay
(Oltenacu *et al.*, 1990; Markusfeld *et al.*, 1990), estrone sulfate analysis (Holdsworth *et al.*,
1982; Warnick *et al.*, 1995), rectal palpation (Hatzidakis *et al.*, 1993), ultrasound (Beal *et al.*,
20 1992; Cameron and Malmo, 1993), and blood tests for pregnancy-specific antigens. Of these,
the progesterone milk assay is the most cost effective for the producer (Oltenacu *et al.*, 1990;
Markusfeld *et al.*, 1990). Next best is rectal palpation, performed at day 50 post-insemination
(Oltenacu *et al.*, 1990).

Even though the prior procedures for pregnancy diagnosis are potentially useful, all have
25 fallen short of expectations in terms of their practical, on-farm use. For example, measurements
of milk or serum progesterone around day 18-22 yield unacceptably high rates of false positives
(Oltenacu *et al.*, 1990; Markusfeld *et al.*, 1990). The presence of estrone sulfate in urine or
serum provides another test, but is only useful after day 100 when concentrations rise
(Holdsworth *et al.*, 1982; Warnick *et al.*, 1995).

30 The discovery of pregnancy-specific protein B (PSP-B) (Butler *et al.*, 1982) provided a
new approach to pregnancy diagnosis since it could be detected in the blood of pregnant cows by

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the fourth week of pregnancy (Sasser *et al.*, 1986; Humblot *et al.*, 1988). Two other groups have developed immunoassays that may be based on an identical or immunologically similar antigen (Zoli *et al.*, 1992a; Mialon *et al.*, 1993; Mialon *et al.*, 1994). In one case, the antigen ($M_r \sim 67$ kDa) was called bovine pregnancy-associated glycoprotein (boPAG; now boPAG-1) (Zoli *et al.*, 1992a); in the second, it was designated as pregnancy serum protein 60 (PSP60) (Mialon *et al.*, 1993; Mialon *et al.*, 1994). The immunoassay for PSP-B/boPAG1/PSP60 has two advantages. First, it can detect pregnancy relatively early. Second, interpretation of the assays does not require knowledge of the exact date of service, since boPAG-1 immunoreactive molecules are always present in the maternal serum of pregnant cows by day 28, and concentrations increase as pregnancy advances (Sasser *et al.*, 1986; Mialon *et al.*, 1993; Mialon *et al.*, 1994).

There remain, however, two major disadvantages to this procedure. First, positive diagnosis in the fourth week of pregnancy remains somewhat uncertain because antigen concentrations in blood are low and somewhat variable. Second, boPAG1 concentrations rise markedly at term (Sasser *et al.*, 1986; Zoli *et al.*, 1992a; Mialon *et al.*, 1993) and, due to the long circulating half-life of the molecule (Kiracofe *et al.*, 1993), the antigen can still be detected 80-100 day postpartum (Zoli *et al.*, 1992a; Mialon *et al.*, 1993; Mialon *et al.*, 1994; Kiracofe *et al.*, 1993), compromising pregnancy diagnosis in cows bred within the early postpartum period. Thus, the test can be carried out in dairy cows at day 30 only if artificial insemination ("AI") is performed at 45-70 days post-partum.

Analysis of other BoPAGs in particular has exhibited potential for use in pregnancy testing. However, such tests can yield high false positive rates. This error rate occurs because the PAG test is done at day 25 of pregnancy. However, some embryos die between day 20 and 30 of pregnancy. This dying tissue can probably produce some PAG. Thus, the cow is PAG positive, but the embryo is dead. The results of this can be a false positive rate that may be unacceptable within some commercial breeding programs. There is, therefore, a need for pregnancy tests with improved accuracy.

It has been shown, in bovines, that shortly after conception one of the dominant proteins released by the conceptus (at about 13 to 20 of pregnancy is interferon-tau, originally called trophoblastin or trophoblast protein-1) (Hansen *et al.*, 1999, reviewed by Roberts *et al.* 1990). After treating endometrial explants from day 18 pregnant cows Naivar *et al.* (Naivar *et al.*, 1995) identified a 17 kDa uterine protein that was released into the medium in response to both

recombinant IFN-tau and recombinant IFN-alpha. The protein was found to be similar in size to human IFN-stimulated gene product-15 (also called huUCRP) and to be immunoreactive with anti-serum against ubiquitin. This protein has, therefore, been termed bovine ubiquitin cross-reactive protein (bUCRP) (Austin *et al.* 1996).

5 Thus, bovine ubiquitin cross-reactive protein (also known as bovine IFN-stimulated gene product-17, ISG17) is produced by uterine endometrium in response to interferon-tau which is secreted by bovine conceptus from day 13 to day 20 of pregnancy. During the pre-implantation period IFN-tau, secreted by the trophoblast layer of the conceptus, functions as a maternal signal denoting recognition of pregnancy in ruminants.

10 Molecular cloning and sequencing of IFN-tau has shown that it belongs to Type I IFN class of proteins (IFN-alpha and IFN-beta). IFN alpha and IFN beta also interact with Type I receptors on the cellular membranes and utilize similar signal transduction pathways. Receptors for IFN-tau are present on the endometrial epithelial cells of the uterus. Sequencing and binding studies have shown that these IFN-tau receptors belong to IFN Type I receptor class.

15 Activation of IFN receptors by the binding of IFN results in biochemical changes within the cell. It also results in the synthesis and secretion of several proteins. Approximately 70 genes have been shown to regulated following IFN alpha treatment in human cell culture models (Schlaak *et al.*, 2002). In Mouse and human cells, IFN-stimulated gene product (ISG15) or ubiquitin cross-reactive protein (UCRP) is one of the earliest responses of type I interferon treatment. ISG15 was the first example of small class of ubiquitin-like proteins that includes
20 SUMO-1, Nedd8 and ubl1.

Bovine ISG17 (or bUCRP) is a bovine ortholog of human ISG15. Sequence comparison showed that bUCRP has 60% sequence identity with human ISG15 (*see*, Hansen *et al.*, 1997).

25 Hansen *et al.*, 1997 reports the pregnancy specific secretion of ISG17, by the endometrium from days 15 to 26 of pregnancy in response to conceptus-derived IFN-tau. In a related study, it has also been reported that the ovine uterus also produces UCRP in response to interferon-tau treatment (Johnson *et al.*, 1999). Finally, it has been suggested that it is possible that ISG17 may be released into peripheral circulation via the histotroph (also known as uterine milk) which nourishes the embryo until the placenta forms. (Austin *et al.*, 1996; Pru *et al.*, 2000)

30 Despite the foregoing, there yet exists a need in the art for a pregnancy test that can be carried out reliably and early in pregnancy which could provide definitive indication as to

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whether re-breeding or culling is required. In general, AI (artificial insemination) is successful less than 50% of the time in cattle and the producer must either rely on overt behavioral signals of return to estrus (that are easily missed) or delay re-breeding until pregnancy failure is confirmed by one of the methods described above. Such delays are extremely costly and constitute a major economic loss to the industry. Consequently, there is thus a need for a feasible, economic, sensitive, and accurate pregnancy test for cattle, and other ruminants, that has low levels of false positive results and yet is sensitive enough to detect pregnancy (or more properly the absence thereof) early enough to allow a herd manager to inseminate the animal immediately following the first missed estrous cycle.

SUMMARY OF THE INVENTION

The invention provides methods for the early detection of pregnancy in livestock such as ungulates (e.g., hoofed animals), and specifically in ruminants such as cows, sheep and goats. In one aspect of the invention, methods are provided for the early detection of pregnancy in a bovine animal comprising: (a) obtaining a sample from the bovine animal; (b) measuring the level of ubiquitin cross-reactive protein (UCRP), the bovine form of which is also known as interferon-stimulated gene product-17 (ISG17); or (c) measuring the level of UCRP and measuring the level of progesterone in the sample, wherein elevated levels ISG17 and progesterone indicate that the bovine animal is pregnant. The sample may be from any biological material, including saliva, serum, blood, milk or urine. In certain embodiments of the invention, the sample may be obtained from the animal at days 16 to 28 post-insemination, including about day 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, or 28, post-insemination:

In one embodiment of the invention, the UCRP that is analyzed is present at increased early pregnancy (*i.e.* between about day 16 and about day 28) and then decreases to constitutive levels shortly thereafter, returning to pre-pregnancy levels by approximately day 28 and afterward.

The measuring may comprise immunologic detection, including detecting a UCRP with polyclonal antisera. In another embodiment of the invention, the polyclonal antisera is prepared against immuno-reactive fragments of a UCRP, such as ISG17, or comprises detecting a UCRP with a monoclonal antibody preparation.

Immunologic detection may be carried out using any technique, including ELISA, RIA, and Western blot. The ELISA may comprise a sandwich ELISA comprising binding of a UCRP

to a first antibody preparation fixed to a substrate and a second antibody preparation labeled with an enzyme. In one embodiment, the enzyme is alkaline phosphatase or horseradish peroxidase.

In certain embodiments of the invention, an elevated level of UCRP that is detected is selected as a cut-off for determination that the animal is pregnant is selected so as to provide a
5 minimal number of false-positive and/or false-negative results while simultaneously providing early detection in accordance with the present invention. The cut-off level of UCRP selected for determination that an animal is pregnant is preferably from at least about 0.5 ng/ml to about 30 ng/ml. Typically, the value is not higher than 20 ng/ml and more preferably it is the level is not higher than 10 ng/ml. In a particularly preferred aspect of this embodiment the serum UCRP
10 cut-off level is between about 1.0 and 5.0 ng/ml. In the most preferred embodiment the cut-off value is 2.0 ng/ml or more.

In another embodiment, measuring UCRP levels may comprise, for example, nucleic acid hybridization, including Northern blotting and nucleic acid hybridization comprises amplification. The amplification may comprise RT-PCR.

15 In the method, measuring progesterone levels may also comprise immunologic detection. In certain embodiments of the invention, immunologic detection may comprise detecting progesterone with polyclonal antisera or detecting progesterone with a monoclonal antibody preparation.

Immunologic detection may be carried out using any technique, including ELISA, RIA,
20 and Western blot. The ELISA may comprise a sandwich ELISA comprising binding of a progesterone to a first antibody preparation fixed to a substrate and a second antibody preparation labeled with an enzyme. In one embodiment, the enzyme is alkaline phosphatase or horseradish peroxidase. In certain embodiments of the invention, the level of UCRP considered to be "elevated" in pregnant animals as compared with non-pregnant animals may range from
25 about 0.5 to about 20 ng/ml of serum, including from about 2 ng/ml to about 10 ng/ml. However, typically basal levels in non-pregnant animals range from 0.5 to 2 ng/ml, whereas the level in pregnant animals is from 2 to 20 ng/ml.

The elevated level of progesterone that is detected may, in certain embodiments of the invention, comprise about 2-5 ng/ml of serum. In particularly preferred aspects of this
30 embodiment the elevated level of progesterone is about 3 ng/ml of serum.

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In certain embodiments of the invention, a sample is obtained from a bovine animal between about day 16 and about day 28 post-insemination, and the elevated levels of UCRP are those greater than about 2 ng/ml of serum and elevated levels of progesterone include those of about 2-5 ng/ml of serum (preferably about 3 ng/ml of serum) or greater. In an more particular aspect of this embodiment the sample is obtained between about day 18 and about day 23 after insemination. In an even more particular aspect of this embodiment the sample is obtained between about day 18 and about day 20. A positive control sample may also be obtained from a pregnant bovine animal, as may a negative control sample from a non-pregnant bovine animal. The method may further comprise measuring UCRP and progesterone levels from a second sample from the bovine animal at a second point in time.

In another embodiment, the invention provides a method of making a breeding decision for a bovine animal comprising: (a) obtaining a sample from the bovine animal, wherein the bovine animal is suspected of being pregnant; (b) measuring the level of ISG17 in the sample; and (c) measuring the level of progesterone in the sample, wherein: (i) elevated levels of ISG17 and progesterone indicate that the bovine animal is pregnant, and no further steps need be taken; (ii) non-elevated levels of ISG17 and progesterone indicate that the bovine animal is not pregnant, and should be injected with gonadotropin-releasing hormone (GnRH), and about seven days later, injected with prostaglandin $F_{2\alpha}$ (PGF), followed by re-insemination; (iii) elevated levels of ISG17 and non-elevated levels of progesterone indicate that the bovine animal is not pregnant due to early embryo death and should be injected with GnRH, and about seven days later, injected with PGF, followed by re-insemination; or (iv) non-elevated levels of ISG17 and elevated levels of progesterone indicate that the bovine animal is not pregnant, and should be injected with PGF, followed by re-insemination. The method may also further comprise steps (ii), (iii) and (iv), about 48 hours after PGF injection and before re-insemination, administering a second injection of GnRH. The method may also further comprise, prior to step (a), inseminating the bovine animal.

Various embodiments contemplate methods for the diagnosis of pregnancy in ruminants and methods for making breeding decisions in ruminants which comprise the detection of the levels of a UCRP (such as ISG17), in a biological sample, either by itself, or in combination with a second antigen (the second antigen may be progesterone or some other pregnancy-specific antigen).

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by
5 reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

Figure 1 shows the typical results of tests of the present invention carried out on both pregnant and open cows.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

10 The invention overcomes the limitations of the prior art by providing a reliable test for early pregnancy diagnosis and methods for use thereof. A reliable yet simple pregnancy test for cattle has long been sought. Typical prior test have either not allowed early detection of pregnancy or have suffered from a high incidence of false positive or false negative results. The prior tests, although potentially useful, have thus fallen short of expectations in terms of their
15 practical, on-farm use. As a result of these shortcomings the animals remain "open" for two or three estrous cycles. It present invention minimizes this "open" time by allowing for the determination of pregnancy, or non-pregnancy, as early as the first estrus following a breeding attempt.

Various embodiments of the present invention overcome the limitations of the prior art
20 by detecting the presence and level of ubiquitin cross-reactive protein (UCRP) (the bovine version of which is also known of as interferon-stimulated gene product-17, or ISG17, the nucleotide sequence of the mRNA encoding the bovine ISG17 protein, as well as the sequence of the protein is provided *infra* in the section entitled Sequence Listing) in a biological sample of an animal suspected of being pregnant. The UCRP may be analyzed by itself to determine the
25 pregnancy status of the animal suspected of being pregnant. In a particularly preferred aspect of this embodiment of the invention the UCRP levels are analyzed immunologically by detecting the UCRP levels with an antibody which was raised to full-length UCRP (either native or recombinant protein). In an even more preferred aspect of this embodiment the antibody specifically recognizes full length UCRP protein.

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Alternatively, in other embodiments of the present invention the UCRP levels present in the biological samples may be determined in conjunction with the determination of the levels of one, or more other pregnancy specific compounds.

In one preferred embodiment the levels of UCRP are determined in conjunction with a
5 determination of the progesterone levels in a biological sample from the same animal in order to provide for the early and accurate diagnoses of pregnancy in bovine and other ruminants. In particular, the inventor has found that by assaying for both progesterone and UCRP, early pregnancy diagnosis is possible with a high degree of accuracy. This is because the combined test measures a conceptus-stimulated component, UCRP, and a maternal component,
10 progesterone, both of which are elevated upon the establishment of a successful pregnancy in cattle ruminant species.

The finding is significant because pregnancy diagnosis is an important component in reproductive management of livestock, particularly in the dairy industry where a high proportion of artificial inseminations fail and additional days open reduce the net operating income to the
15 producer.

Various embodiments of the present invention comprise a method and means (including kits) for the diagnosis of pregnancy or non-pregnancy in an animal suspected to be in the early stages of pregnancy. In particular aspects of these embodiments the subject animal is a ruminant selected from the group consisting of bovine, ovine, and caprine. In an even more particular
20 aspect of this embodiment the animal is bovine. In the most preferred aspect of this embodiment the animal is a dairy heifer or dairy cow.

In some embodiments of the present invention the diagnosis of pregnancy or non-pregnancy is made by analyzing a biological sample from the animal suspected of being in the early stages of pregnancy. The analysis is done by detecting the presence and/or amount of
25 ubiquitin cross-reactive protein (UCRP) and progesterone in the biological sample from animals, including bovines, suspected of being in the early stages pregnancy. In various embodiments the biological sample comprises serum, plasma, blood, saliva, urine, milk, or any other suitable sample from the subject animal which is compatible with the present invention. In one particularly preferred embodiment the biological sample is from blood, serum, or plasma.

30 In various embodiments of the invention the biological sample is obtained from the animal suspected of being pregnant from 15 to 28 days after natural breeding or artificial

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insemination has occurred. Preferably, the sample may be collected at any of days 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, or 28 after breeding. More preferably the sample may be collected at about days 16 to 23 after breeding, and even more preferably 18-21 days after breeding.

5 In various embodiments of the present invention the animal whose pregnancy status is to be determined is a ruminant. More preferably the animal is a bovine, ovine, or caprine animal. Even more preferably the animal is bovine and most preferably the animal is a beef or dairy cow.

In various embodiments of the invention, the pregnancy test is carried out by detecting UCRP and/or progesterone by immunological methods. For example, by using monoclonal or
10 polyclonal antibodies raised against intact UCRP (or alternatively antibodies against peptides comprising immunoreactive epitopes of UCRP). In an preferred aspect of this embodiment the antibodies (monoclonal or polyclonal) are raised against either full-length native or full-length recombinant UCRP. In an even more preferred embodiment the full length UCRP is full length ISG17. Most preferably the immunogen is full-length bovine ISG17 and the antibodies are
15 monoclonal antibodies.

For immunological progesterone analysis, commercially available assay kits are available that may be used to measure serum levels of progesterone. Using a UCRP assay and the commercial progesterone assay, it was found that pregnancy detection could be performed as early as about day 17 in cattle or during peri-implantation in ruminants, generally. Additionally,
20 the present invention is beneficial in that it results in a very low rate (<5%) of false positive and false negative results.

According to the present invention, the present inventors have determined that UCRP in combination with progesterone can be used advantageously expressed in early stages of pregnancy and, therefore, can be used as markers in the detection of pregnancy at an early stage.
25 In cattle, UCRP may be used individually or in combination with other diagnostic methods to provide a diagnostic evaluation of pregnancy. In particular the UCRP, bISG17 may be used. It is envisioned that UCRPs from other species, will also prove useful, alone or in combination, for similar purposes.

As noted above, the presence of detectable ISG17 above background levels in the
30 maternal circulation indicates the presence of a conceptus in the uterus. However, the circulating levels of ISG17 will diminish when the conceptus ceases to produce IFN-tau, which typically

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occurs at the end of the implantation process (after approximately day 21 of pregnancy). The conceptus begins to produce IFN-tau from about days 13 to 21 of pregnancy, with production peaking at around days 16 to 18. The raise in serum ISG17 levels then follows in response to the secreted IFN-tau. A raise in ISG17 can be observed beginning at about day 15 of pregnancy, with levels peaking at about days 18 to 20. Thereafter, the ISG17 level begins to decline.

The serum progesterone concentration in the pregnant cows is usually about 3 ng/ml or above on day 16 and continues to rise, thereafter. If a cow is not pregnant, the serum progesterone concentration decline significantly from day 16 onwards and drops to below 3 ng/ml (in most cases below 1.0 ng/ml) by day 19 to day 23 (Santos *et al.*, 2002; Ayalon, 1978; Weibold, 1988).

Chandrasekaran *et al.*, 1990 have shown that levels of progesterone in the milk correlate with serum progesterone levels, although they are three-fold lower, suggesting that analysis of milk progesterone levels is also applicable for use according to the instant invention.

One embodiment of the present invention is directed to a method comprising a combined testing for ISG17 and progesterone used to determine the pregnancy status of a cow. According to one particular aspect of this embodiment, the presence of ISG17 above a background threshold level (e.g. >2 ng/ml) with serum progesterone value of from about 2-5 ng/ml or above, the cow is likely to indicate that a cow is pregnant. In an especially preferred aspect of this embodiment the serum progesterone level is about 3 ng/ml or higher. In contrast, if ISG17 is absent in the serum or present at a level below the "threshold level" and the animal has a serum progesterone values below about 2-5 ng/ml, and preferably below about 3 ng/ml, the animal is considered to be not pregnant or "open."

The specificity of pregnancy diagnosis with the combination testing would likely improve when testing is done from day 16 through day 23. The reason being, serum progesterone values of ~80% of open dairy cows are below 2 ng/ml from days 20 to 23.

ISG-17 (above baseline)+ Progesterone 3ng/ml or above	Pregnancy positive
ISG17- Progesterone below 3ng/ml	Pregnancy negative

The specificity and sensitivity of the combined test is likely to range from 75 to 95% when testing is done from day 16 to day 23 following artificial insemination.

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The specificity and sensitivity of the combine test would be 70 to 90% due to a high incidence of early embryonic loss compared to day 45 pregnancy status (palpation data).

Various embodiments of the present invention comprise methods for detecting the levels of UCRP and/or progesterone that are well known and widely used in the art. Particular aspects of this embodiment include determination of UCRP and/or progesterone levels using immunological and/or nucleic acid based methods.

A. Immunologic Detection of UCRP and Progesterone

Contemplated embodiments of the present invention include those employing the use of antibodies in the immunologic detection of UCRP and/or progesterone. Various useful immunodetection methods have been described in the scientific literature, such as, *e.g.*, Nakamura *et al.* (1987; incorporated herein by reference). Immunoassays, in their most simple and direct sense, are binding assays. Certain preferred embodiments include the use of immunoassays including various types of enzyme linked immunosorbent assays (ELISAs) and radioimmunoassays (RIA). Immunohistochemical detection using tissue sections also is contemplated as useful for the present invention. However, it will be readily appreciated that detection is not limited to such techniques, and Western blotting, dot blotting, FACS analyses, and the like also may be used in connection with the present invention.

In general, immunobinding methods include obtaining a sample suspected of containing a protein, peptide or antibody, and contacting the sample with an antibody or protein or peptide in accordance with the present invention, as the case may be, under conditions effective to allow the formation of immunocomplexes. Preferred samples, according to the present invention, are fluids, such as milk, urine, blood, serum or saliva.

Contacting the selected biological sample with the protein, peptide or antibody under conditions effective and for a period of time sufficient to allow the formation of immune complexes (primary immune complexes) is generally a matter of simply adding the composition to the sample and incubating the mixture for a period of time long enough for the antibodies to form immune complexes with UCRP or progesterone. After this time, the UCRP or progesterone antibody mixture will be washed to remove any non-specifically bound antibody species, allowing only those antibodies specifically bound within the primary immune complexes to be detected.

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In general, the detection of immunocomplex formation is well known in the art and may be achieved through the application of numerous approaches. These methods are generally based upon the detection of a label or marker, such as any radioactive, fluorescent, biological or enzymatic tags or labels of standard use in the art. U.S. Patents concerning the use of such labels
5 include 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241, each incorporated herein by reference. Of course, one may find additional advantages through the use of a secondary binding ligand such as a second antibody or a biotin/avidin ligand binding arrangement, as is known in the art.

Usually, the primary immune complexes may be detected by means of a second binding
10 ligand that has binding affinity for the UCRP or progesterone-specific first antibody. In these cases, the second binding ligand may be linked to a detectable label. The second binding ligand is itself often an antibody, which may thus be termed a "secondary" antibody. The primary immune complexes are contacted with the labeled, secondary binding ligand, or antibody, under conditions effective and for a period of time sufficient to allow the formation of secondary
15 immune complexes. The secondary immune complexes are then generally washed to remove any non-specifically bound labeled secondary antibodies or ligands, and the remaining label in the secondary immune complexes is then detected.

Further methods include the detection of primary immune complexes by a two step approach. A second binding ligand, such as an antibody, that has binding affinity for the UCRP
20 or progesterone antibody is used to form secondary immune complexes, as described above. The second binding ligand contains an enzyme capable of processing a substrate to a detectable product and, hence, amplifying signal over time. After washing, the secondary immune complexes are contacted with substrate, permitting detection.

Progesterone can also be detected in accordance with the invention using various
25 commercially available detection kits. For example, the COAT-A-COUNT™ progesterone kit used by the inventors, which is available from Diagnostics Products Corporation (Los Angeles, CA). Examples of other assays that have been described include the immunoenzymatic technique described, for example, by Stefanakis *et al.*, (1994) and by Stanley *et al.* (1986); and salivary progesterone level assays described, for example, by Lu *et al.*, (1997) and Vienravi *et al.*, 1994.
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B. ELISA

In one particularly preferred embodiment of the present invention the UCRP detected is bovine ISG17 and the bISG17 and the progesterone are detected using monoclonal antibodies to either bISG17 or progesterone as part of an ELISA.

5 Thus as a part of the practice of the present invention, the widely known and well-understood principles of an enzyme-linked immunoassay (ELISA) may be used. ELISA was first introduced by Engvall and Perlmann (1971) and has become a powerful analytical tool using a variety of protocols (Engvall, 1980; Engvall, 1976; Engvall, 1977; Gripenberg *et al.*, 1978; Sarngadharan *et al.*, 1984). ELISA allows for substances to be passively adsorbed to solid
10 supports such as plastic to enable facile handling under laboratory conditions. For a comprehensive treatise on ELISA the skilled artisan is referred to "ELISA; Theory and Practice" (Crowther, 1995 incorporated herein by reference).

The sensitivity of ELISA methods is dependent on the turnover of the enzyme used and the ease of detection of the product of the enzyme reaction. Enhancement of the sensitivity of
15 these assay systems can be achieved by the use of fluorescent and radioactive substrates for the enzymes. Immunoassays encompassed by the present invention include, but are not limited to those described in U.S. Patent 4,367,110 (double monoclonal antibody sandwich assay) and U.S. Patent 4,452,901 (western blot). Other assays include immunoprecipitation of labeled ligands and immunocytochemistry, both *in vitro* and *in vivo*.

20 In a particularly preferred embodiment, the invention comprises a "sandwich" ELISA, where anti-UCRP antibodies, preferably anti-bISG17 antibodies, are immobilized onto a selected surface, such as a well in a polystyrene microtiter plate or a dipstick. Then, a test composition suspected of containing UCRP, *e.g.*, a clinical sample, such as blood or serum, is contacted with the surface. After binding and washing to remove non-specifically bound immunocomplexes,
25 the bound antigen may be detected by a second antibody to the UCRP.

In another exemplary ELISA, polypeptides from the sample are immobilized onto a surface and then contacted with the anti-UCRP antibodies. After binding and washing to remove non-specifically bound immune complexes, the bound antibody is detected. Where the initial antibodies are linked to a detectable label, the primary immune complexes may be
30 detected directly. Alternatively, the immune complexes may be detected using a second

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antibody that has binding affinity for the first antibody, with the second antibody being linked to a detectable label.

Another ELISA in which the UCRP is immobilized involves the use of antibody competition in the detection. In this ELISA, labeled antibodies are added to the wells, allowed to
5 bind to the UCRP, and detected by means of their label. The amount of UCRP in a sample is determined by mixing the sample with the labeled antibodies before or during incubation with coated wells. The presence of UCRP in the sample acts to reduce the amount of antibody available for binding to the well, and thus reduces the ultimate signal.

Irrespective of the format employed, ELISAs have certain features in common, such as
10 coating, incubating or binding, washing to remove non-specifically bound species, and detecting the bound immune complexes. In coating a plate with either antigen or antibody, one will generally incubate the wells of the plate with a solution of the antigen or antibody, either overnight or for a specified period of hours. The wells of the plate will then be washed to remove incompletely adsorbed material. Any remaining available surfaces of the wells are then
15 "coated" with a nonspecific protein that is antigenically neutral with regard to the test antisera. These include bovine serum albumin (BSA), casein and solutions of milk powder. The coating allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.

In ELISAs, it is probably more customary to use a secondary or tertiary detection means
20 rather than a direct procedure. Thus, after binding of a protein or antibody to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the control human cancer and/or clinical or biological sample to be tested under conditions effective to allow immune complex (antigen/antibody) formation. Detection of the immune complex then requires a labeled secondary binding ligand
25 or antibody, or a secondary binding ligand or antibody in conjunction with a labeled tertiary antibody or third binding ligand.

"Under conditions effective to allow immune complex (antigen/antibody) formation" means that the conditions preferably include diluting the antigens and antibodies with solutions such as BSA, bovine gamma globulin (BGG), evaporated or powdered milk, and phosphate
30 buffered saline (PBS)/TWEENTM. These added agents also tend to assist in the reduction of nonspecific background.

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The "suitable" conditions also mean that the incubation is at a temperature and for a period of time sufficient to allow effective binding. Incubation steps are typically from about 1 h to 2 h to 4 h, at temperatures preferably on the order of 25°C to 27°C, or may be overnight at approximately 4°C.

5 To provide a detecting means, the second or third antibody will have an associated label to allow detection. Preferably, this will be an enzyme that will generate color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the first or second immune complex with a urease, glucose oxidase, alkaline phosphatase or hydrogen peroxidase-conjugated antibody for a period of time and under
10 conditions that favor the development of further immune complex formation (*e.g.*, incubation for 2 h at room temperature in a PBS-containing solution such as PBS-TWEENTM).

After incubation with the labeled antibody, and subsequent to washing to remove unbound material, the amount of label is quantified, *e.g.*, by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azido-di-(3-ethyl-benzthiazoline-6-sulfonic
15 acid [ABTS] and H₂O₂, in the case of peroxidase as the enzyme label. Quantitation is then achieved by measuring the degree of color generation, *e.g.*, using a visible spectra spectrophotometer.

A variant of ELISA is the enzyme-linked coagulation assay, or ELCA (U.S. Patent 4,668,621), which uses the coagulation cascade combined with the labeling enzyme RVV-XA as
20 a universal detection system. The advantage of this system for the current invention, is that the coagulation reactions can be performed at physiological pH in the presence of a wide variety of buffers. It is therefore possible to retain the integrity of complex analytes.

C. Nucleic Acid Detection

In some embodiments of the instant invention, it will be desirable to detect nucleic acids
25 (mRNAs or cDNAs) which encode UCRPs, such as bISG17, and/or proteins involved in the biosynthesis of progesterone to determine the levels of the corresponding proteins. Such methods include Northern assays and RT-PCR. The following describe methods relevant to the detection and quantification of such nucleic acids.

1. Hybridization

30 The use of a probe or primer of between 13 and 100 nucleotides, preferably between 17 and 100 nucleotides in length, or in some aspects of the invention up to 1-2 kilobases or more in length,

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allows the formation of a duplex molecule that is both stable and selective. Molecules having complementary sequences over contiguous stretches greater than 20 bases in length are generally preferred, to increase stability and/or selectivity of the hybrid molecules obtained. One will generally prefer to design nucleic acid molecules for hybridization having one or more
5 complementary sequences of 20 to 30 nucleotides, or even longer where desired. Such fragments may be readily prepared, for example, by directly synthesizing the fragment by chemical means or by introducing selected sequences into recombinant vectors for recombinant production.

Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of DNAs and/or RNAs or to
10 provide primers for amplification of DNA or RNA from samples. Depending on the application envisioned, one would desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of the probe or primers for the target sequence.

For applications requiring high selectivity, one will typically desire to employ relatively high stringency conditions to form the hybrids. For example, relatively low salt and/or high
15 temperature conditions, such as provided by about 0.02 M to about 0.10 M NaCl at temperatures of about 50°C to about 70°C. Such high stringency conditions tolerate little, if any, mismatch between the probe or primers and the template or target strand and would be particularly suitable for isolating specific genes or for detecting specific mRNA transcripts. It is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide.

20 Conditions may be rendered less stringent by increasing salt concentration and/or decreasing temperature. For example, a medium stringency condition could be provided by about 0.1 to 0.25 M NaCl at temperatures of about 37°C to about 55°C, while a low stringency condition could be provided by about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Hybridization conditions can be readily manipulated depending on the desired results.

25 In other embodiments, hybridization may be achieved under conditions of, for example, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 1.0 mM dithiothreitol, at temperatures between approximately 20°C to about 37°C. Other hybridization conditions utilized could include approximately 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, at temperatures ranging from approximately 40°C to about 72°C.

30 In certain embodiments, it will be advantageous to employ nucleic acids of defined sequences of the present invention in combination with an appropriate means, such as a label, for

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determining hybridization. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of being detected. In preferred embodiments, one may desire to employ a fluorescent label or an enzyme tag such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmentally undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known that can be employed to provide a detection means that is visibly or spectrophotometrically detectable, to identify specific hybridization with complementary nucleic acid containing samples.

In general, it is envisioned that the probes or primers described herein will be useful as reagents in solution hybridization, as in PCR™, for detection of expression of corresponding genes, as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to hybridization with selected probes under desired conditions. The conditions selected will depend on the particular circumstances (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, *etc.*). Optimization of hybridization conditions for the particular application of interest is well known to those of skill in the art. After washing of the hybridized molecules to remove non-specifically bound probe molecules, hybridization is detected, and/or quantified, by determining the amount of bound label. Representative solid phase hybridization methods are disclosed in U.S. Patents 5,843,663, 5,900,481 and 5,919,626. Other methods of hybridization that may be used in the practice of the present invention are disclosed in U.S. Patents 5,849,481, 5,849,486 and 5,851,772. The relevant portions of these and other references identified in this section of the Specification are incorporated herein by reference.

2. Amplification of Nucleic Acids

Nucleic acids used as a template for amplification may be isolated from cells, tissues or other samples according to standard methodologies (Sambrook *et al.*, 1989). In certain embodiments, analysis is performed on whole cell or tissue homogenates or biological fluid samples without substantial purification of the template nucleic acid. The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to first convert the RNA to a complementary DNA.

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The term "primer," as used herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty and/or thirty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded and/or single-stranded form, although the single-stranded form is preferred.

Pairs of primers designed to selectively hybridize to nucleic acids corresponding to a UCRP (*e.g.*, bISG17) are contacted with the template nucleic acid under conditions that permit selective hybridization. Depending upon the desired application, high stringency hybridization conditions may be selected that will only allow hybridization to sequences that are completely complementary to the primers. In other embodiments, hybridization may occur under reduced stringency to allow for amplification of nucleic acids contain one or more mismatches with the primer sequences. Once hybridized, the template-primer complex is contacted with one or more enzymes that facilitate template-dependent nucleic acid synthesis. Multiple rounds of amplification, also referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

The amplification product may be detected or quantified. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of incorporated radiolabel or fluorescent label or even via a system using electrical and/or thermal impulse signals (Affymax technology; Bellus, 1994).

A number of template dependent processes are available to amplify the oligonucleotide sequences present in a given template sample. One of the best known amplification methods is the polymerase chain reaction (referred to as PCRTM) which is described in detail in U.S. Patents 4,683,195, 4,683,202 and 4,800,159, and in Innis *et al.*, 1988, each of which is incorporated herein by reference in their entirety.

A reverse-transcriptase coupled PCRTM amplification procedure may be performed to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known (see Sambrook *et al.*, 1989). Alternative methods for reverse transcription utilize thermostable DNA polymerases. These methods are described in WO 90/07641. Polymerase chain reaction methodologies are well known in the art. Representative methods of RT-PCR are described in U.S. Patent 5,882,864.

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Another method for amplification is ligase chain reaction ("LCR"), disclosed in European Application No. 320 308, incorporated herein by reference in its entirety. U.S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence. A method based on PCRTM and oligonucleotide ligase assay (OLA), disclosed in U.S. Patent 5,912,148, may also be used.

Alternative methods for amplification of target nucleic acid sequences that may be used in the practice of the present invention are disclosed in U.S. Patent Nos. 5,843,650, 5,846,709, 5,846,783, 5,849,546, 5,849,497, 5,849,547, 5,858,652, 5,866,366, 5,916,776, 5,922,574, 5,928,905, 5,928,906, 5,932,451, 5,935,825, 5,939,291 and 5,942,391, GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety.

Qbeta Replicase, described in PCT Application No. PCT/US87/00880, may also be used as an amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence which may then be detected.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[alpha-thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention (Walker *et al.*, 1992). Strand Displacement Amplification (SDA), disclosed in U.S. Patent 5,916,779, is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR (Kwoh *et al.*, 1989; Gingeras *et al.*, PCT Application WO 88/10315, incorporated herein by reference in their entirety). European Application No. 329 822 disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention.

PCT Application WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter region/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the

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resultant RNA transcripts. Other amplification methods include "race" and "one-sided PCR" (Frohman, 1990; Ohara *et al.*, 1989).

3. Detection of Nucleic Acids

Following any amplification, it may be desirable to separate the amplification product from the template and/or the excess primer. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods (Sambrook *et al.*, 1989). Separated amplification products may be cut out and eluted from the gel for further manipulation. Using low melting point agarose gels, the separated band may be removed by heating the gel, followed by extraction of the nucleic acid.

Separation of nucleic acids may also be effected by chromatographic techniques known in art. There are many kinds of chromatography which may be used in the practice of the present invention, including adsorption, partition, ion-exchange, hydroxylapatite, molecular sieve, reverse-phase, column, paper, thin-layer, and gas chromatography as well as HPLC.

In certain embodiments, the amplification products are visualized. A typical visualization method involves staining of a gel with ethidium bromide and visualization of bands under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the separated amplification products can be exposed to x-ray film or visualized under the appropriate excitatory spectra.

In one embodiment, following separation of amplification products, a labeled nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, or another binding partner carrying a detectable moiety.

In particular embodiments, detection is by Southern blotting and hybridization with a labeled probe. The techniques involved in Southern blotting are well known to those of skill in the art (see Sambrook *et al.*, 1989). One example of the foregoing is described in U.S. Patent 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

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Other methods of nucleic acid detection that may be used in the practice of the instant invention are disclosed in U.S. Patents 5,840,873, 5,843,640, 5,843,651, 5,846,708, 5,846,717, 5,846,726, 5,846,729, 5,849,487, 5,853,990, 5,853,992, 5,853,993, 5,856,092, 5,861,244, 5,863,732, 5,863,753, 5,866,331, 5,905,024, 5,910,407, 5,912,124, 5,912,145, 5,919,630, 5,925,517, 5,928,862, 5,928,869, 5,929,227, 5,932,413 and 5,935,791, each of which is incorporated herein by reference.

4. Kits

All the essential materials and/or reagents required for detecting UCRP and/or progesterone in a sample may be assembled together in a kit. This generally will comprise a probe or primers designed to hybridize specifically to individual nucleic acids and/or antibodies capable of specifically recognizing the molecules of interest in the practice of the present invention. Also included may be enzymes suitable for detecting the interaction of the antibodies with the target antigens and/or enzymes for amplifying nucleic acids, including various polymerases (reverse transcriptase, Taq, *etc.*), deoxynucleotides and buffers to provide the necessary reaction mixture for amplification. Such kits may also include enzymes and other reagents suitable for detection of specific proteins/compounds or nucleic acids or amplification products and/or for detecting antibody/ligand interaction. Such kits generally will comprise, in suitable means, distinct containers for each individual reagent or enzyme as well as for each probe, primer pair, and/or antibody.

20 I. Livestock Breeding Programs

One advance of the current invention is that it allows early detection of pregnancy with a low incidence of false positive results. Early detection of pregnancy is important because it allows re-breeding of animals found to not be pregnant, thereby minimizing the period during which the animal is "open." A low incidence of false positives is necessary to allow implementation of an effective re-breeding protocol. Prior pregnancy tests typically either were unable effectively detect early pregnancy or exhibited high incidence of false positives.

A type of early pregnancy test which has been used is the detection of pregnancy associated antigens (PAGs). An advantage of this test is that it can be done at day 25 of pregnancy. However, some embryos die between day 20 and 30 of pregnancy and, in some cases, the dying tissue may produce PAG. Thus cows may be PAG positive but the embryo is dead.

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As discussed herein below, the inventors have found that by analyzing progesterone levels in addition to UCRP, a very low incidence (<5%) of false positives can be obtained. This is because the corpus luteum regresses shortly after embryo death. Thus, a cow with a dying embryo would have ISG17 but low progesterone. Because the progesterone is an absolute
5 requirement for establishing pregnancy, a cow with low serum progesterone cannot maintain pregnancy.

Another class of embodiments of the present invention provides for methods of making breeding decisions for animals. Preferably, the subject animals are ruminants, including bovine, ovine, and caprine. More preferably, the animals are beef or dairy cattle.

10 The method comprises obtaining a biological sample from an animal suspected of being pregnant. Preferably, the biological sample comprises a blood, serum, plasma, milk, urine, or saliva sample, but any other biological samples suitable for use with the present invention are also contemplated. In the case of cattle the sample is preferably collected 16-28 days after natural breeding or artificial insemination (collectively, "breeding") has taken place. More
15 preferably the sample is serum collected 16-23 days after breeding and most preferably the sample is collected 18-21 days after breeding. Next, the sample is analyzed to determine the levels of UCRP (preferably bISG17 for cattle) and progesterone present in the sample. If the level of UCRP and progesterone are both elevated, as compared with non-pregnant controls, the animal is determined to be pregnant and no further action need be taken.

20 If the levels of neither UCRP nor progesterone are elevated, this indicates that the animal is not pregnant and additional actions are required. First, the animal should be injected with gonadotropin-releasing hormone (GnRH), and about seven days later, injected with prostaglandin $F_{2\alpha}$ (PGF), followed by re-insemination.

If the level of UCRP is elevated but the levels of progesterone are not elevated, this
25 indicates that the animal is not pregnant, due to early embryo death or a failure in maternal recognition of pregnancy. In this instance the animal should be injected with GnRH, then about seven days later, injected with PGF, followed by re-insemination.

If the level of UCRP is not elevated, but the level of progesterone is elevated, this indicates that the animal is not pregnant. The animal should be injected with PGF, followed by
30 re-insemination.

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In preferred aspects of this embodiment of the invention, those where either or both the UCRP and progesterone levels are not elevated the method further comprises administering a (second) dose of GnRH about 48 hours after the PGF injection and before re-insemination.

In one particularly preferred embodiment of the present invention the PGF injection is administered at day 20 post-insemination and re-insemination is carried out at day 28 post-insemination.

A. Estrus and ovulation

Dairy cows come into estrus once every 21 days. Cows display characteristic behaviors during estrus. Dairy workers can identify cows in estrus by these characteristic behaviors. Cows ovulate an egg about 28 hours after the onset of estrus. Most dairy cows are inseminated artificially about 12 hours after the onset of estrus so that sperm are in the reproductive tract when the cow ovulates.

B. Efficiency of reproduction in dairy cows

Lactating dairy cows are watched for estrus. They are inseminated when they come into estrus so that they can become pregnant and have another calf. The efficiency with which cows are detected in estrus is low. Only about 50% of cows in estrus are actually detected by farmers. Of the cows detected in estrus and inseminated, only about 30% will become pregnant. Thus, only about 15% (50% x 30%) of ovulations result in a pregnancy. Thus, dairy reproduction can be inefficient because cows in estrus are not always seen and those inseminated do not always get pregnant. Although most cows could theoretically be artificially inseminated once every 21 days, the true insemination interval on farms is typically once every 40 to 60 days. This lost time results in negative impact on dairy income because the extended "open" period reduces the economic efficiency as a result of the extended period between calves and the reduced milk production during the phase just prior to drying (dairy cattle reach peak lactation 85 to 115 days post partum). Thus, it is more profitable if the cattle become pregnant as soon as possible. Furthermore, the efficiency of reproduction has worsened since 1985 because of consolidation of the dairy industry. Larger farms means and labor shortages have resulted in a reduction in the amount of time that the cattle interact with humans and a consequential reduction in the detection of estrus. This is a significant problem as dairy reproduction is the key to the dairy farm's success. In fact to most common reason for the culling of is that they do not become pregnant and are considered barren.

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C. Corpus luteum and progesterone

After a cow ovulates a corpus luteum (CL) is formed on the ovary and the CL secretes the hormone progesterone. Progesterone, can be detected in the blood of the mother is needed to maintain the pregnancy. If the egg is fertilized and the embryo grows and survives then the corpus luteum will be maintained until the end of gestation (280 days). If the egg is not fertilized or the embryo dies then the corpus luteum will regress and the cow will reenter estrus.

D. UCRP and pregnancy testing

Following conception the conceptus produces interferon-tau (IFN-tau). IFN-tau stimulates the uterine endometrium to produce UCRP, which can be detected in the blood of the mother at about 15 to 28 days of pregnancy in bovine and other ruminants. The UCRP pregnancy test is designed to detect UCRP in the blood/serum of the mother. A pregnant cow will also have high progesterone in blood because she will have a corpus luteum. Thus, pregnant cows will have UCRP and progesterone in their blood/serum.

E. Pregnancy testing in dairy

The problem with reproductive management in dairy cattle is that pregnancy detection has previously typically been done 35 to 60 days after breeding. Furthermore, most non-pregnant cows are simply injected with PGF because the status of the corpus luteum is not known. However, the pregnancy tests of the current invention can be done 19 to 46 days sooner than these traditional pregnancy testing and only cows with a CL need be injected with PGF. Cows that do not have a CL (and will not respond to PGF) should, instead be injected with GnRH and then treated with PGF at the appropriate time. By implementing this plan, farmers will know which cows are pregnant and inseminate non-pregnant cows within about 28 days of their first insemination. The 16-25 day interval from breeding to pregnancy detection is shorter than current methods and the 28-day interval from first breeding to second breeding for non-pregnant cows is much shorter than the industry average.

Pregnancy testing in dairy cows has usually been done by rectal palpation (manually feeling for an embryo in the uterus). The manual test is typically done 35 to 60 days after breeding. On large dairies, a veterinarian is often employed 100% time to do manual pregnancy testing. The only alternative to manual testing is ultrasound testing. While, ultrasound testing can be done at 28 days after breeding, it is not routine because the equipment is expensive and testing takes longer than rectal palpation.

F. Drugs used to manipulate reproductive cycles in dairy

Dairy cows can be injected with prostaglandin $F_{2\alpha}$ (PGF) to regress the corpus luteum and cause estrus. PGF only has effect if the cow has a corpus luteum. Cows that do not have a corpus luteum do not respond to PGF. Instead, dairy cows without a corpus luteum can be
 5 injected with gonadotropin-releasing hormone (GnRH) to cause ovulation and the formation of a corpus luteum. One typical method for managing dairy cows without a corpus luteum is to inject GnRH, wait 7 days (allows CL to form), inject PGF and await the cow's next estrus. Both PGF and GnRH are inexpensive and are commonly used in dairy herds (either alone or in combination). Another approach is to inject GnRH, wait seven days and inject PGF, and then
 10 wait two days and inject GnRH. This protocol (Ovsynch protocol) is popular because cows can be inseminated after the second GnRH without the need for estrus detection.

G. Implementation of Improved Pregnancy Tests in Breeding Programs

Using the new assays, there are four possible outcomes with respect to the UCRP and progesterone results: +/+, +/-, -/+ and -/-. Based on the results, various steps will be desired for
 15 implementation of breeding programs. The different possibilities and the likely desired course of action are set forth below in Table 1.

Table 1: Reproductive plan implemented 21 days after breeding

UCRP Test Result	Progesterone Test Result	Pregnancy outcome	Farmer action
Positive	Positive	Cow is pregnant	No further action required.
Positive	Negative	The embryo underwent early embryonic death and the cow is not pregnant.	Cow does not have a CL (based on low progesterone). Inject GnRH (cause ovulation), wait seven days, inject PGF (regress CL). The farmer can breed at estrus or an alternative would be to give another injection of GnRH at 48 hours after PGF to induce ovulation and breed. These are common reproductive management treatments in dairy.
Negative	Positive	Cow is not pregnant	Cow has a CL but does not have an embryo. Inject PGF to regress the CL. The farmer can breed at estrus or an alternative would be give another injection of GnRH at 48 hours after PGF to induce ovulation and breed. These are common reproductive management treatments in dairy
Negative	Negative	Cow is not pregnant	Cow does not have a CL and does not have an embryo. Inject GnRH, wait

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			seven days, inject PGF. The farmer can breed at estrus or an alternative would be give another injection of GnRH at 48 hours after PGF to induce ovulation and breed. These are common reproductive management treatments in dairy.
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H. Types of Assays used

According to various embodiments of the present invention the assays to detect the levels of progesterone may be carried out either on biological samples collected on the same day or on biological samples collected on different days, as is convenient and/or most efficacious. In a preferred aspect of the of this embodiment the assays to determine the levels of UCRP and progesterone levels are determined in a sample or samples which are collected on the same day.

In a particularly preferred aspect of this embodiment the assays are ELISA or another antibody based assay wherein the antibodies function separately. For example, they may be located at separate positions on the assay device or the assay may utilize one separate device for UCRP and another for progesterone. In an even more preferred aspect of this embodiment of the invention the method for determination of the UCRP and progesterone levels in the biological samples comprises the use of one or more "Lateral Flow Assay" device(s) wherein the two antigens are detected separately.

According to the instant invention a "Lateral Flow Assay" means an immunochromatographic determination of the presence or absence of an antigen in a biological sample from an animal by: a) combining the sample with a coloring agent-coupled antibody, specific for the antigen; b) allowing the resulting combination to migrate into a first region containing a second antibody to the antigen, which is not coupled to a coloring agent so that the appearance of color in the first region indicates that the antigen is present in the sample; and c) allowing the combination to migrate from the first region into a second region containing an antibody to the first antibody, so that the appearance of color in the second region, together with the absence of color in the first region, serves as a control which indicates that the antibody to the antigen is present, but the antigen is not present.

II. The Endometrium and Ubiquitin Cross-Reactive Protein isolation

The placenta is the hallmark of the eutherian mammal. Rather than being the most anatomically conserved mammalian organ, however, it arguably is the most diverse (Haig, 1993). Placentation ranges from the invasive hemochorial type, as in the human, where the

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trophoblast surface is in direct contact with maternal blood, to the epitheliochorial (*e.g.*, pig), where the uterine epithelium is not eroded (Amoroso, 1952). Not only is placental structure highly variable, the polypeptide hormones the placenta produces also vary between species (Haig, 1993; Roberts *et al.*, 1996). For example, no group of mammals other than higher
5 primates possesses a chorionic gonadotrophin homologous to hCG for luteal support in early pregnancy, and only the ruminant ungulates are known to produce Type I interferon as an antiluteolytic hormone (Roberts *et al.*, 1996). In response to interferon-tau, the endometrial luminal and glandular epithelial cells secrete several proteins including UCRP into the uterine lumen (reviewed in 'Biology of progesterone action during pregnancy recognition and
10 maintenance of pregnancy' Spencer TE and Bazer FW., *Frontiers in Bioscience* 7:1879-1898, 2002). Uterine flushings or endometrial tissue collected from days 16 to day 25 pregnant animals are used for purification of IFN-tau induced proteins (Hansen et al., *Endocrinology* 138:5079-5082, 1997).

A. Purification of the Proteins

15 It may be desirable to purify the ISG17 and other UCRPs for use as the antigen for the generation of polyclonal or monoclonal antibodies, or for other reasons. Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level, the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Having separated the polypeptide from other proteins, the polypeptide of interest may be further purified
20 using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion chromatography; polyacrylamide gel electrophoresis; isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even HPLC.

25 Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an encoded protein or peptide. The term "purified protein or peptide" as used herein, is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state. A purified protein or peptide therefore also refers to a protein or peptide, free
30 from the environment in which it may naturally occur.

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Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a "-fold purification number" (*i.e.*, 2-fold, 5-fold, 10-fold, 50-fold, 100-fold, 1000-fold, *etc.*). The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulfate, PEG, antibodies and the like or by heat or acid pH denaturation of contaminating proteins, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater "-fold" purification than

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the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE and according to how extensively it is glycosylated (Capaldi *et al.*, 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

High Performance Liquid Chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain an adequate flow rate. Separation can be accomplished in a matter of min, or at most an hour. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

Gel chromatography, or molecular sieve chromatography, is a special type of partition chromatography that is based on molecular size. The theory behind gel chromatography is that the column, which is prepared with tiny particles of an inert substance that contain small pores, separates larger molecules from smaller molecules as they pass through or around the pores, depending on their size. As long as the material of which the particles are made does not adsorb the molecules, the sole factor determining rate of flow is the size. Hence, molecules are eluted from the column in decreasing size, so long as the shape is relatively constant. Gel chromatography is unsurpassed for separating molecules of different size because separation is independent of all other factors such as pH, ionic strength, temperature, *etc.* There also is virtually no adsorption, less zone spreading and the elution volume is related to molecular weight.

Affinity Chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule that it can specifically bind to. This is a receptor-ligand type interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (alter pH, ionic strength, temperature, *etc.*).

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A particular type of affinity chromatography useful in the purification of carbohydrate containing compounds is lectin affinity chromatography. Lectins are a class of substances that bind to a variety of polysaccharides and glycoproteins. Lectins are usually coupled to agarose by cyanogen bromide. Concanavalin A coupled to Sepharose was the first material of this sort to be used and has been widely used in the isolation of polysaccharides and glycoproteins other lectins that have been include lentil lectin, wheat germ agglutinin which has been useful in the purification of N-acetyl glucosaminyl residues and *Helix pomatia* lectin. Lectins themselves are purified using affinity chromatography with carbohydrate ligands. Lactose has been used to purify lectins from castor bean and peanuts; maltose has been useful in extracting lectins from lentils and jack bean; N-acetyl-D galactosamine is used for purifying lectins from soybean; N-acetyl glucosaminyl binds to lectins from wheat germ; D-galactosamine has been used in obtaining lectins from clams and L-fucose will bind to lectins from lotus.

The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand. One of the most common forms of affinity chromatography is immunoaffinity chromatography. The generation of antibodies that would be suitable for use in accord with the present invention is discussed below.

B. Antigen Compositions

The present invention provides for the use of a UCRP (such as bISG17) or peptides fragments thereof as antigens for the generation of polyclonal antisera and monoclonal antibodies for use in the detection of UCRP in a biological sample as part of a test for the diagnosis of pregnancy. It is envisioned that some a UCRP, or portions thereof, will be coupled, bonded, bound, conjugated or chemically-linked to one or more agents *via* linkers, polylinkers or derivatized amino acids. This may be performed such that a bispecific or multivalent composition or vaccine is produced. It is further envisioned that the methods used in the preparation of these compositions will be familiar to those of skill in the art and should be suitable for administration to animals, *i.e.*, pharmaceutically acceptable. Preferred agents are the carriers such as keyhole limpet hemocyanin (KLH) or glutathione-S-transferase.

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In order to formulate UCRP for immunization, one will generally employ appropriate salts and buffers to render the polypeptides stable. Aqueous compositions of the present invention comprise an effective amount of an UCRP (such as bISG17) antigen to the host animal, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium.

5 Such compositions may be referred to as inocula. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and

10 the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the vectors or cells of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The compositions of the present invention may include classic pharmaceutical preparations. Administration of these compositions according to the present invention will be

15 *via* any common route so long as the target tissue is available *via* that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration may be by orthotopic, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions,

20 described *supra*.

The UCRP may be administered parenterally or intraperitoneally. Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary

25 conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy

30 syringability exists. It should be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and

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fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle
5 size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of
10 agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the PAGs in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion
15 medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The compositions of the present invention may be formulated in a neutral or salt form.
20 Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such
25 organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile
30 aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl

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solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, preparations should meet applicable sterility, pyrogenicity, general safety and purity standards.

III. Generating Antibody Preparations Which are Reactive With UCRPs or Progesterone

Various embodiments of the present invention contemplate the generation of an antibody preparation wherein the antibodies therein are immunoreactive with UCRP (or an immunoreactive portion thereof) or progesterone.

In various aspects of this embodiment of the invention it is contemplated that antibodies specific for UCRP, or a fragment thereof, may be raised against UCRP from any ruminant animal. In a more preferred aspect of this embodiment of the invention the UCRP is from a bovine, ovine, or caprine animal. In an even more preferred aspect of this embodiment the UCRP is bovine ISG17 or a fragment thereof. In one particularly preferred aspect of this embodiment of the invention the bovine ISG17 used to as the antigen elicit the antibodies is recombinant ISG17 obtained using a published DNA sequence for ISG17 (such as the one provided in the Sequence Listing of this disclosure) and recombinant DNA techniques which are well known to those of ordinary skill in the art.

Such an antibody preparation can be a polyclonal or a monoclonal antibody composition, both of which are contemplated for use with preferred embodiments of the present invention. Means for preparing and characterizing antibodies are well known in the art (see, *e.g.*, Harlow and Lane, 1988). While it is possible to generate a polyclonal antibody preparation which simultaneously comprises some antibodies recognizing UCRP and some recognizing progesterone. The preferred embodiment of the present invention is to generate antibodies to UCRP and progesterone separately.

Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a peptide or polypeptide of the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera.

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Typically an animal used for production of anti-antisera is a non-human animal including rabbits, mice, rats, hamsters, pigs or horses. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

Antibodies, both polyclonal and monoclonal, specific for isoforms of antigen may be prepared using conventional immunization techniques, as will be generally known to those of skill in the art. A composition containing antigenic epitopes of the compounds of the present invention can be used to immunize one or more experimental animals, such as a rabbit or mouse, which will then proceed to produce specific antibodies against the compounds of the present invention. Polyclonal antisera may be obtained, after allowing time for antibody generation, simply by bleeding the animal and preparing serum samples from the whole blood.

It is proposed that the monoclonal antibodies of the present invention will find useful application in standard immunochemical procedures, such as ELISA and Western blot methods and in immunohistochemical procedures such as tissue staining, as well as in other procedures which may utilize antibodies specific for UCRR-related antigen epitopes. Additionally, it is proposed that monoclonal antibodies specific to the particular UCRP of different species may be utilized in other useful applications.

In general, both polyclonal and monoclonal antibodies against UCRP or progesterone may be used in a variety of embodiments. For example, they may be employed in antibody cloning protocols to obtain cDNAs or genes encoding antibodies to UCRP and/or proteins involved in the biosynthesis of progesterone. They may also be used in inhibition studies to analyze the effects of UCRP related peptides or progesterone related compounds in cells or animals. Anti-UCRP or antibodies to progesterone pathway enzymes will also be useful in immunolocalization studies to analyze the distribution of UCRP or enzymes that participate in progesterone biosynthesis or metabolism during various cellular events, for example, to determine the cellular or tissue-specific distribution of UCRP or progesterone biosynthesis or metabolism under different points in the cell cycle. A particularly useful application of such antibodies is in purifying native or recombinant UCRP, for example, using an antibody affinity column. The operation of all such immunological techniques will be known to those of skill in the art in light of the present disclosure.

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Means for preparing and characterizing antibodies are well known in the art (see, e.g., Harlow and Lane, 1988; incorporated herein by reference). More specific examples of monoclonal antibody preparation are give in the examples below.

As is well known in the art, a given composition may vary in its immunogenicity. It is
5 often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means
for conjugating a polypeptide to a carrier protein are well known in the art and include
10 glutaraldehyde, *m*-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As also is well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant (a
15 non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*), incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal,
20 intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster, injection may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate
25 mAbs.

MAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, e.g., a purified or partially purified UCRP or progesterone. The immunizing composition is
30 administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep or frog cells is also possible.

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The use of rats may provide certain advantages (Goding, 1986), but mice are preferred, with the BALB/c mouse being most preferred as this is most routinely used and generally gives a higher percentage of stable fusions.

Following immunization, somatic cells with the potential for producing antibodies, specifically B-lymphocytes (B-cells), are selected for use in the MAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmoblast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5×10^7 to 2×10^8 lymphocytes.

The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render them incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, 1986; Campbell, 1984). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, P3-X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with cell fusions.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 ratio, though the ratio may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described (Kohler and Milstein, 1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter *et al.*, (1977). The use of electrically induced fusion methods is also appropriate (Goding, 1986).

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Fusion procedures usually produce viable hybrids at low frequencies, around 1×10^{-6} to 1×10^{-8} . However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, *e.g.*, hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B-cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B-cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide mAbs. The cell lines may be exploited for mAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide mAbs in high concentration. The individual cell lines could also be cultured *in vitro*, where the mAbs are naturally secreted into the culture medium from which they can be readily

obtained in high concentrations. mAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography.

IV. Examples

5 The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate
10 that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1: Development of an Immunoassay for ISG17

 An immunoassay which is developed using antibodies raised against full-length, native or recombinant UCRP offers more specificity than one using antibodies raised to peptide fragments
15 of UCRP. This is because antibodies raised to whole protein antigen are more likely to be conformation-specific antibodies and offer more specificity to immunoassay. In contrast, antibodies raised against peptide fragment antigens are more likely to be sequence specific rather than conformation specific and will, consequently, be more likely to cross-react with related and/or unrelated proteins thereby diminishing the immunoassay's specificity and/or sensitivity.

20 An immunoassay developed with full-length, native or recombinant bovine ISG17 will detect bISG17 protein present in a biological sample such as serum, plasma, saliva, urine, or blood with high specificity and sensitivity. A bISG17 specific immunoassay, may be developed by first raising an antibody to either purified native ISG17, such as bISG17, isolated from endometrial secretions. Alternatively, purified recombinant ISG17 produced in yeast or bacteria
25 may be used as the antigen. Recombinant protein produced in yeast has been shown to have proper folding by a bioassay (Pru et al., 2000). However, production of bISG17 in bacteria may require re-folding and the extent of re-folding could be tested with a bioassay described by Pru et al (2000).

Example 3: Polyclonal UCRP assay development

30 The following section describes the means for a polyclonal antibody based assay development for UCRP. The assay may be standardized using purified recombinant bISG17 as

antigen and affinity purified rabbit polyclonal antibodies. This assay may be used to determine the feasibility of detecting UCRP during early pregnancy using various polyclonal antibodies.

Antigen proteins may be prepared and isolated by any means known to those of ordinary skill in the art.

5 Antibodies may be generated in rabbits according to the standard protocol-using purified UCRP in Freund's complete adjuvant. After a two-week interval these rabbits may be boosted with the antigen with incomplete adjuvant. The rabbits can then be boosted every two weeks until sufficient antisera were collected and stored at -70 °C. Polyclonal antibodies can then be affinity purified using protein A chromatography and dialyzed in PBS. Purified antibodies are
10 then aliquoted and stored at -70 °C.

Example 3: Design of a combination UCRP/progesterone early pregnancy test and Results expected.

Due to high levels of false positive results obtained using a UCRP assay only, a new assay format analyzing both progesterone and UCRP levels may be designed. Additionally, due
15 to a high incidence of early embryonic loss in cattle, a combined testing of pregnancy induced protein (UCRP) and progesterone (maternal component) would able to more accurately predict the pregnancy status of an animal than would testing for only one analyte. A series of designated UCRP and progesterone cutoff levels for pregnancy determination based on given concentrations of UCRP and progesterone can be formulated. For each day post insemination as serious of
20 analyses may be done to determine the appropriate cut off levels for serum (or other sample) levels of UCRP in cows.

For example a studies may be done which determine the serum levels of UCRP and progesterone in a statistically significant number of cows on the days following artificial insemination or sham-insemination as control. By charting the serum UCRP and progesterone
25 levels in those cattle, subsequently identified as being pregnant by another method (such as rectal palpation or sonography) a standard may be statistically calculated which provides a base line level of UCRP and progesterone, below which a cow should be considered non-pregnant and above which a cow should be considered pregnant.

Using these values a test can be made which provide the appropriate indication of a
30 positive result for UCRP and/or progesterone only when the levels of those compounds in the biological sample are above the determined level.

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The same data, may also be used to determine the optimal window to allow for the early detection of pregnancy which provides both acceptable sensitivity and accuracy and diagnosis early enough to allow re-breeding so as to minimize the time the cow is open.

For example two cutoff ranges (3 ng/ml and 2 ng/ml) have been determined for
5 progesterone. The cutoff ranges were selected based on: a) pregnancy history of the cows, b) Progesterone levels during estrus cycle and pregnancy in cows.

It is expected that the results achieved by the method described above will provide a combination UCRP/progesterone pregnancy detection test which is both accurate and sensitive. Furthermore, this test will provide critical information allowing herd managers to optimize
10 profits by minimizing the amount of time that herd animals remain open.

Example 4: Diagnosis of Pregnancy/Non-pregnancy in a bovine suspected of being pregnant.

Antibodies may be raised respectively against bISG17 and progesterone. This antibodies may then be employed to prepare a kit comprising, *inter alia*, the components necessary to
15 provide sandwich-type ELISA assays for individually detecting the presence bISG17 and progesterone in a biological sample.

The pregnancy status of a bovine may then be determined by collecting a biological sample, such as serum from the cow 20 days after natural breeding or artificial insemination has taken. The biological sample can then be analyzed to determine the levels of bISG17 and
20 progesterone in the sample. Threshold levels can be individually established which will be determined to be a "positive" result (a positive result meaning that the animal is pregnant) for bISG17 and progesterone ELISA assays. According to one aspect of the present invention it has been determined that for certain applications a suitable cutoff level for designation as a "positive result" for bISG17 is 2 ng/ml or more and for progesterone the level is 3 ng/ml or more.

25 The results of the bISG17 and progesterone assays can be determined and a diagnosis of pregnant or "open" can be made in accordance with the results shown in Figure 1.

Example 5: Resynchronization of Dairy Cows and Heifers after UCRP/Progesterone**Pregnancy Diagnosis**

The following a method for re-breeding cows and heifers that are diagnosed as not pregnant after a bISG17/progesterone test. Generally, cattle are tested for UCRP/progesterone 16 to 28 days after natural breeding or artificial insemination and are diagnosed pregnant or non-pregnant. The resynchronization method is implemented on non-pregnant cows 0 to 2 days after the ISG17/progesterone test. Animals are treated in the following sequence: (i) inject prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; a hormone causing regression of the corpus luteum); (ii) wait two days, inject gonadotropin releasing hormone (GnRH; a hormone causing ovulation); (iii) wait 0 to 8 hours, (iv) inseminate artificially.

Methods. Dairy cows and heifers are tested for ISG17 16 to 28 days after first artificial insemination. Cattle diagnosed not pregnant are treated with 5 mL Lutalyse (25 mg $PGF_{2\alpha}$), two days later were treated with 2 mL Cystorelin (100 μ g GnRH), and are inseminated 0 to 8 hours after GnRH. The resynchronization treatment is administered 0 to 2 days after the ISG17 test (16 to 30 days after first insemination). Pregnancy is determined 30 to 60 days after insemination.

While the methods and kits of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and kits described herein and in the steps or in the sequence of steps of the method described herein without departing from the inventive concept of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference:

- U.S. Patent 3,817,837
- 5 U.S. Patent 3,850,752
- U.S. Patent 3,939,350
- U.S. Patent 3,996,345
- U.S. Patent 4,196,265
- U.S. Patent 4,275,149
- 10 U.S. Patent 4,277,437
- U.S. Patent 4,366,241
- U.S. Patent 4,367,110
- U.S. Patent 4,452,901
- U.S. Patent 4,668,621
- 15 U.S. Patent 4,683,195
- U.S. Patent 4,683,202
- U.S. Patent 4,800,159
- U.S. Patent 4,883,750
- U.S. Patent 5,279,721
- 20 U.S. Patent 5,840,873
- U.S. Patent 5,843,640
- U.S. Patent 5,843,650
- U.S. Patent 5,843,651
- U.S. Patent 5,843,663
- 25 U.S. Patent 5,846,708
- U.S. Patent 5,846,709
- U.S. Patent 5,846,717
- U.S. Patent 5,846,726
- U.S. Patent 5,846,729
- 30 U.S. Patent 5,846,783
- U.S. Patent 5,849,481

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- U.S. Patent 5,849,486
- U.S. Patent 5,849,487
- U.S. Patent 5,849,497
- U.S. Patent 5,849,546
- 5 U.S. Patent 5,849,547
- U.S. Patent 5,851,772
- U.S. Patent 5,853,990
- U.S. Patent 5,853,992
- U.S. Patent 5,853,993
- 10 U.S. Patent 5,856,092
- U.S. Patent 5,858,652
- U.S. Patent 5,861,244
- U.S. Patent 5,863,732
- U.S. Patent 5,863,753
- 15 U.S. Patent 5,866,331
- U.S. Patent 5,866,366
- U.S. Patent 5,882,864
- U.S. Patent 5,900,481
- U.S. Patent 5,905,024
- 20 U.S. Patent 5,910,407
- U.S. Patent 5,912,124
- U.S. Patent 5,912,145
- U.S. Patent 5,912,148
- U.S. Patent 5,916,776
- 25 U.S. Patent 5,916,779
- U.S. Patent 5,919,626
- U.S. Patent 5,919,630
- U.S. Patent 5,922,574
- U.S. Patent 5,925,517
- 30 U.S. Patent 5,928,862
- U.S. Patent 5,928,869

- U.S. Patent 5,928,905
U.S. Patent 5,928,906
U.S. Patent 5,929,227
U.S. Patent 5,932,413
5 U.S. Patent 5,932,451
U.S. Patent 5,935,791
U.S. Patent 5,935,825
U.S. Patent 5,939,291
U.S. Patent 5,942,391
10 U.S. App. 09/273,164
Amoroso, In: *Marshall's Physiology of Reproduction*, Parkes (Ed.), Little Brown and Co., Boston, 2:127-311, 952, 1952.
Atkinson *et al.*, *J. Biol. Chem.*, 268(35):26679-26685, 1993.
Austin *et al.*, *Endocrine*, 5:191-197, 1996.
15 Austin *et al.*, *Biology of Reproduction*, 54:600-606, 1996.
Ayalon, NJ, *J. Reprod. Fert.* 54:483-493, 1978.
Beal *et al.*, *J. Anim. Sci.*, 70:924-929, 1992.
Bellus, *J. Macromol. Sci. Pure Appl. Chem.*, A31(1): 1355-1376, 1994.
Butler *et al.*, *Biol. Reprod.*, 26:925-933, 1982.
20 Cameron and Malmo, *Austr. Vet. J.*, 70:109-111, 1993.
Campbell *et al.*, *J. Mol. Biol.*, 180:1-19, 1984.
Capaldi *et al.*, *Biochem. Biophys. Res. Comm.*, 76:425, 1977.
Chagas e Silva, *Theriogenology*, 58:51-59 (2002).
Chandrasekaran *et al.*, *Indian Veterinary Journal*, 67:87-87, 1990.
25 Crowther, In: *Methods in Molecule Biology*, Vol. 42, Humana Press; New Jersey, 1995.
Davies, *Ann. Rev. Bioiphs. Chem.*, 19:189-215, 1990.
D'Cunha *et al.*, *J. Immunology*, 157:4100-4108, 1996.
Engvall and Perlmann, *Immunochem.*, 8:871-873, 1971.
Engvall, *Lancet*, 2(8000):1410, 1976.
30 Engvall, *Med Biol.*, 55(4):193-200, 1977.
Engvall, *Methods Enzymol*, 70(A):419-39, 1980.

- European App. 329 822
European App. 320 308
Frohman, In: *PCR Protocols: A Guide To Methods And Applications*, Academic Press, N.Y., 1990.
- 5 GB App. 2 202 328
Gefter *et al.*, *Somatic Cell Genet.*, 3:231-236, 1977.
Goding, In: *Monoclonal Antibodies: Principles and Practice*, 2d ed., Academic Press, Orlando, Fl, pp 60-61, 71-74, 1986.
Gripenberg *et al.*, *Scand J Immunol.*, 7(2):151-7, 1978.
- 10 Guillomot, *J. Reprod. Fertil.*, 49(Suppl):39-51, 1995.
Guruprasad *et al.*, *Protein Engin.*, 9:949-856, 1996.
Haig, *Rev. Biol.*, 68:495-532, 1993.
Hansen *et al.* *Endocrinology*, 138:5079-5082, 1997
Hansen, *et al.*, *J. Reproduction and Fertility Supplement*, 54:329-339, 1999.
- 15 Harlow and Lane, In: *Antibodies, a Laboratory Manual*, Cold Spring Harbor Laboratory, pp 139-281, 1988.
Hatzidakis *et al.*, *J. Reprod. Fertil.*, 98:235-240, 1993.
Holdsworth *et al.*, *J. Endocrin.*, 95:7-12, 1982.
Humblot *et al.*, *Theriogenol.*, 30:257-268, 1988.
- 20 Johnson *et al.* *Biol. Reproduction*, 61:312-318, 1999.
King *et al.*, *J. Reprod. Fertil.*, 59:95-100, 1980.
Kiracofe *et al.*, *J. Anim. Sci.*, 71:2199-2205, 1993.
Kohler and Milstein, *Eur. J. Immunol.*, 6:511-519, 1976.
Kohler and Milstein, *Nature*, 256:495-497, 1975.
- 25 Kwoh *et al.*, *Proc. Nat. Acad. Sci. USA*, 86: 1173, 1989.
Markusfeld *et al.*, *Br. Vet. J.*, 146: 504-508, 1990.
Mialon *et al.*, *Reprod. Nutr. Dev.*, 33:269-282, 1993.
Mialon *et al.*, *Reprod. Nutr. Dev.*, 34:65-72, 1994.
Naivar *et al.*, *Biology of Reproduction*, 52:848-854, 1995.
- 30 Nakamura *et al.*, In: *Handbook of Experimental Immunology* (4th Ed.), Weir *et al.* (Eds.), Blackwell Scientific Publ., Oxford, 1:27, 1987.

-46-

- Ohara *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:5673-5677, 1989.
- Oltenacu *et al.*, *J. Dairy Sci.*, 73:2826-2831, 1990.
- Patel *et al.*, *Theriogenol.*, 44:827-833, 1995.
- PCT App. PCT/US87/00880
- 5 PCT App. PCT/US89/01025
- PCT App. PCT/US90/07641
- PCT App. WO 88/10315
- PCT App. WO 89/06700
- PCT App. No. _____ filed 11/20/02 and claiming priority from U.S. Provisional 60/331,882.
- 10 Pru *et al.*, *Biology of Reproduction*, 63:619-628, 2000.
- Remington's Pharmaceutical Sciences*, 15th ed., pp 1035-1038 and 1570-1580.
- Rippe *et al.*, *Mol. Cell Biol.*, 10:689-695, 1990.
- Roberts *et al.*, *Endocrine Reviews*, 13:432-452, 1990.
- Roberts *et al.*, *Biol. Reprod.*, 54:294-302, 1996.
- 15 Roberts *et al.*, *Prog. Nucl. Acid Res. Mol. Biol.*, 56:287-326, 1996.
- Sambrook *et al.*, *In: Cold Spring Harbor Laboratory Press*, 2nd Ed., 1989.
- Santos, JEP *et al.*, *Journal of Dairy Science*, vol. 85 Supplement 1, pp. 265, 2002.
- Sarnagadharan *et al.*, *Princess Takamatsu Symp.*, 15:301-8, 1984.
- Sasser *et al.*, *Biol. Reprod.* 35:936-942, 1986.
- 20 Sasser *et al.*, *J. Reprod. Fertil.*, 37(Suppl):109-113, 1989.
- Schlaak *et al.*, *J. Biol. Chem.* 277 (51): 49428-49437, 2002
- Spencer, TE and Bazer, FW, *Frontiers in Bioscience*, 7:1879-1898, 2002.
- Stanley *et al.*, *Veterinarian Record*, 664-667, 1986.
- Stefanakis *et al.*, *Bull. Hellenic Vet. Med. Soc.*, 45:37-43, 1994.
- 25 Streenan and Diskin, *In: Embryonic Mortality in Farm Animals*, Sreenan and Diskin (Eds.),
Martinus Nijhoff Publishers, 1-11, 1986.
- Szafranska *et al.*, *Biol. Reprod.*, 53:21-28, 1995.
- Tür-Kaspa *et al.*, *Mol. Cell Biol.*, 6:716-718, 1986.
- Vienravi *et al.*, *J. Med. Assoc. Thai.*, 77(3):138-47, 1994.
- 30 Walker *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:392-396 1992.
- Warnick *et al.*, *Theriogenol.*, 44:811-825, 1995.

-47-

- Wiebold, JL, *J. Reprod. Fert.* 84:393-399, 1988.
- Wooding *et al.*, *Placenta*, 13:101-113, 1992.
- Wooding, J. *Reprod. Fertil.*, 62:15-19, 1981.
- Xie *et al.*, *Biol. Reprod.*, 51:1145-1153, 1994.
- 5 Xie *et al.*, *Biol. Reprod.*, 54: 122-129, 1996.
- Xie *et al.*, *Biol. Reprod.*, 57:1384-1393, 1997a.
- Xie *et al.*, *Gene*, 159:193-197, 1995.
- Xie *et al.*, *Proc. Natl. Acad. Sci. USA*, 94:12809-12816, 1997b.
- Xie *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:10247-10251, 1991.
- 10 Zoli *et al.*, *Biol. Reprod.*, 45:1-10, 1991.
- Zoli *et al.*, *Biol. Reprod.*, 46:623-629, 1992b.
- Zoli *et al.*, *Biol. Reprod.*, 46:83-92, 1992a.

-48-

SEQUENCE LISTING**DNA SEQUENCE**

5 PubMedNucleotideProteinGenomeStructurePopSetTaxonomyOMIMBooks
 Search PubMed Protein Nucleotide PopSet Taxonomy Genome OMIM
 Structure Domains GEO Books MapViewDr TestDb UniSTS CDD SNP
 Journals UniGene NCBI Search for
 Limits Preview/Index History Clipboard Details
 10 Summary ASN.1 FASTA TinySeq XML GenBank GBSeq XML GI List Graphics
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 GGGAGGGCTCCCCATGCAGCGCAGTGAATAAAGTTGTAGCAAAGCC (SEQ ID NO:1)

PROTEIN SEQUENCES

30 **I. First Reference:**

PubMedNucleotideProteinGenomeStructurePopSetTaxonomyOMIMBooks
 Search PubMed Protein Nucleotide PopSet Taxonomy Genome OMIM
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 Journals UniGene NCBI Search for
 Limits Preview/Index History Clipboard Details
 Summary ASN.1 FASTA TinySeq XML GI List GenPept GBSeq XML Graphics
 XML default
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 DEFINITION ubiquitin cross-reactive protein [Bos taurus].
 ACCESSION AAB57687
 VERSION AAB57687.1 GI:2098776
 45 DBSOURCE locus BTU96014 accession U96014.1
 KEYWORDS

-49-

SOURCE Bos taurus
 ORGANISM Bos taurus
 Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
 Mammalia; Eutheria; Cetartiodactyla; Ruminantia; Pecora; Bovoidea;
 5 Bovidae; Bovinae; Bos.

REFERENCE 1 (residues 1 to 154)
 AUTHORS Austin,K.J., Pru,J.K. and Hansen,T.R.
 TITLE Complementary Deoxyribonucleic Acid Sequence Encoding Bovine
 Ubiquitin Cross-Reactive Protein: A Comparison with Ubiquitin and a
 10 15-kDa Ubiquitin Homolog
 JOURNAL Endocrine 5, 191-197 (1996)

REFERENCE 2 (residues 1 to 154)
 AUTHORS Austin,K.J., Pru,J.K. and Hansen,T.R.
 TITLE Direct Submission
 15 JOURNAL Submitted (01-APR-1997) Animal Science, University of Wyoming, P.O.
 Box 5684, Laramie, WY 82071, USA

COMMENT Method: conceptual translation supplied by author.

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 /coded_by="U96014.1:77..541"
 /note="ubiquitin homolog; interferon stimulated gene
 product"

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I. Second Reference:

PubMedNucleotideProteinGenomeStructurePopSetTaxonomyOMIMBooks

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LOCUS UCRP_BOVIN 154 aa linear MAM 15-JUN-2002
 DEFINITION Ubiquitin cross-reactive protein (Interferon-stimulated gene
 45 product 17).
 ACCESSION O02741
 VERSION O02741 GI:2501451

-50-

DBSOURCE swissprot: locus UCRP_BOVIN, accession O02741;
class: standard.
created: Nov 1, 1997.
sequence updated: May 30, 2000.
5 annotation updated: Jun 15, 2002.
xrefs: gi: 2098775, gi: 2098776, gi: 5931941, gi: 5931942
xrefs (non-sequence databases): HSSPP02248, InterProIPR000626,
PfamPF00240, PRINTSPR00348, SMARTSM00213, PROSITEPS50053
KEYWORDS Interferon induction; Repeat.
10 SOURCE Bos taurus (cow)
ORGANISM Bos taurus
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
Mammalia; Eutheria; Cetartiodactyla; Ruminantia; Pecora; Bovoidea;
Bovidae; Bovinae; Bos.
15 REFERENCE 1 (residues 1 to 154)
AUTHORS Austin,K.J., Pru,J.K. and Hansen,T.R.
TITLE Complementary deoxyribonucleic acid sequence encoding bovine
ubiquitin cross-reactive protein: a comparison with ubiquitin and a
15-kDa ubiquitin homolog
20 JOURNAL Endocrine 5, 191-197 (1996)
REMARK SEQUENCE FROM N.A.
TISSUE=Endometrium
REFERENCE 2 (residues 1 to 154)
AUTHORS Perry,D.J., Austin,K.J. and Hansen,T.R.
25 TITLE Cloning of interferon-stimulated gene 17: the promoter and nuclear
proteins that regulate transcription
JOURNAL Mol. Endocrinol. 13 (7), 1197-1206 (1999)
MEDLINE 99333127
PUBMED 10406469
30 REMARK SEQUENCE FROM N.A.
REFERENCE 3 (residues 1 to 154)
AUTHORS Austin,K.J., Ward,S.K., Teixeira,M.G., Dean,V.C., Moore,D.W. and
Hansen,T.R.
TITLE Ubiquitin cross-reactive protein is released by the bovine uterus
35 in response to interferon during early pregnancy
JOURNAL Biol. Reprod. 54 (3), 600-606 (1996)
MEDLINE 96432315
PUBMED 8835381
REMARK IDENTIFICATION.
40 REFERENCE 4 (residues 1 to 154)
AUTHORS Johnson,G.A., Austin,K.J., Van Kirk,E.A. and Hansen,T.R.
TITLE Pregnancy and interferon-tau induce conjugation of bovine ubiquitin
cross-reactive protein to cytosolic uterine proteins
JOURNAL Biol. Reprod. 58 (4), 898-904 (1998)
45 MEDLINE 98206795
PUBMED 9546718
REMARK FUNCTION.

-51-

REFERENCE 5 (residues 1 to 154)

AUTHORS Hansen,T.R., Austin,K.J. and Johnson,G.A.

TITLE Transient ubiquitin cross-reactive protein gene expression in the
bovine endometrium

5 JOURNAL Endocrinology 138 (11), 5079-5082 (1997)

MEDLINE 98006468

PUBMED 9348245

REMARK EXPRESSION.

COMMENT -----

10 This SWISS-PROT entry is copyright. It is produced through a
collaboration between the Swiss Institute of Bioinformatics and
the EMBL outstation - the European Bioinformatics Institute.
The original entry is available from <http://www.expasy.ch/sprot>
and <http://www.ebi.ac.uk/sprot>

15 -----

[FUNCTION] PROBABLY ACTS AS A UBIQUITIN BY CONJUGATION TO
INTRACELLULAR TARGET PROTEINS, THROUGH AN ENZYME PATHWAY
DISTINCT FROM THAT OF UBIQUITIN, DIFFERING IN SUBSTRATE SPECIFICITY
AND INTERACTION WITH LIGATING ENZYMES. SPECIFICALLY, IN RESPONSE TO
20 IFN-TAU SECRETED BY THE CONCEPTUS, MAY LIGATE TO AND REGULATE
PROTEINS INVOLVED WITH RELEASE OF PROSTAGLANDIN F2-ALPHA (PGF), AND
THUS PREVENT LYSIS OF THE CORPUS LUTEUM AND MAINTAIN THE
PREGNANCY. AS IT IS ALSO DETECTED IN UTERINE FLUSHINGS, MAY ALSO HAVE
AN EXOCRINE FUNCTION. MAY ALSO, BY INDUCING THE SECRETION OF IFN-
25 GAMMA FROM T-CELLS, AUGMENT NATURAL KILLER (NK) CELL
PROLIFERATION, AND ACTIVATE MONOCYTES AND MACROPHAGES.

[SUBCELLULAR LOCATION] CYTOPLASMIC AND SECRETED.

[TISSUE SPECIFICITY] EXPRESSED IN ENDOMETRIUM OF PREGNANT COW.
NOT DETECTED IN SPLEEN, LIVER, CORPUS LUTEUM OR MUSCLE.

30 [DEVELOPMENTAL STAGE] FOLLOWS THE PATTERN OF EXPRESSION OF
INTERFERON TAU BY THE CONCEPTUS. FIRST APPEARS ON DAY 15 OF
PREGNANCY IN ENDOMETRIUM OF COWS, REACHES A MAXIMUM ON DAY 18
AND REMAINS HIGH THROUGH DAY 26.

[INDUCTION] BY TYPE I INTERFERONS.

35 [SIMILARITY] CONTAINS 2 UBIQUITIN-LIKE DOMAINS.

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	/db_xref="taxon:9913"
40 gene	1..154
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Region	2..78

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WHAT IS CLAIMED IS:

1. A method for the early detection of pregnancy in an animal comprising:
 - (a) obtaining a sample from said animal;
 - (b) measuring the level of an ubiquitin cross-reactive protein (UCRP) in said sample;
 - 5 and
 - (c) measuring the level of progesterone in said sample,wherein elevated levels of UCRP and progesterone indicate that said bovine animal is pregnant.
2. The method of claim 1, wherein said sample is saliva, serum, blood, milk or urine.
- 10 3. The method of claim 2, wherein said sample is serum.
4. The method of claim 1, wherein said sample is obtained from said animal at day 16 to 28 post-insemination.
5. The method of claim 4, wherein said sample is obtained from said animal at day 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, or 28 post-insemination.
- 15 6. The method of claim 4, wherein said sample is obtained from said animal at day 16 to 23 post-insemination.
7. The method of claim 4, wherein said sample is obtained from said animal at day 18 to 21 post-insemination.
8. The method of claim 1 wherein the animal is a ruminant.
- 20 9. The method of claim 8 wherein the ruminant is bovine, ovine, or caprine.
10. The method of claim 8 wherein the ruminant is a beef or dairy cow.
11. The method of claim 1, wherein said measuring UCRP levels comprises immunologic detection.
- 25 12. The method of claim 11, wherein said immunologic detection comprises detecting UCRP with polyclonal antisera.

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13. The method of claim 1 wherein the UCRP is bovine interferon-stimulated gene product-17 (bISG17).
14. The method of claim 13, wherein the immunologic detection comprises detecting bISG17 with a monoclonal antibody preparation.
- 5 15. The method of claim 14, wherein said immunologic detection comprises ELISA.
16. The method of claim 14, wherein said immunologic detection comprises RIA.
17. The method of claim 14, wherein said immunologic detection comprises Western blot.
18. The method of claim 15, wherein said ELISA is a sandwich ELISA comprising binding of a bISG17 to a first antibody preparation fixed to a substrate and a second antibody
10 preparation labeled with an enzyme.
19. The method of claim 18, wherein said enzyme is alkaline phosphatase or horseradish peroxidase.
20. The method claim 3, wherein level of total UCRP is from about 0.5 ng/ml to about 30 ng/ml of serum.
- 15 21. The method of 20 wherein the level of total UCRP is from about 1.0 ng/ml to about 5 ng/ml of serum.
22. The method of 20 wherein the level of total UCRP is at least about 2.0 ng/ml to about 3.0 ng/ml of serum.
23. The method of claim 1, wherein measuring UCRP levels comprises nucleic acid
20 hybridization.
24. The method of claim 23, wherein nucleic acid hybridization comprises Northern blotting.
25. The method of claim 24, wherein nucleic acid hybridization comprises amplification.
26. The method of claim 25, wherein amplification comprises RT-PCR.

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27. The method of claim 1, wherein measuring progesterone levels comprises immunologic detection.
28. The method of claim 27, wherein said immunologic detection comprises detecting progesterone with polyclonal antisera.
- 5 29. The method of claim 27, wherein said immunologic detection comprises detecting progesterone with a monoclonal antibody preparation.
30. The method of claim 27, wherein said immunologic detection comprises ELISA.
31. The method of claim 27, wherein said immunologic detection comprises RIA.
32. The method of claim 27, wherein said immunologic detection comprises Western blot.
- 10 33. The method of claim 30, wherein said ELISA is a sandwich ELISA comprising binding of progesterone to a first antibody preparation fixed to a substrate and a second antibody preparation labeled with an enzyme.
34. The method of claim 33, wherein said enzyme is alkaline phosphatase or horseradish peroxidase.
- 15 35. The method claim 3, wherein elevated level of progesterone is about 2 ng/ml to about 5 ng/ml of serum.
36. The method of claim 35 wherein the level of progesterone is about 3 ng/ml of serum.
37. The method of claim 1, wherein measuring progesterone levels comprises measuring progesterone biosynthesis pathway enzyme levels by nucleic acid hybridization, immunologic detection or enzyme activity measurement.
- 20 38. The method of claim 37, wherein nucleic acid hybridization comprises Northern blotting.
39. The method of claim 37, wherein nucleic acid hybridization comprises amplification.
40. The method of claim 39, wherein amplification comprises RT-PCR.

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41. The method of claim 3, wherein said sample is obtained at day 20 post-insemination, and the elevated levels of UCRP and progesterone are at least about 0.5 to 30 ng/ml and about 2 to about 5 ng/ml, respectively.
42. The method of claim 41 wherein the level of UCRP is at least about 1.0 ng/ml to about 5.0 ng/ml of serum.
43. The method of claim 41 wherein the level of UCRP is at least about 2.0 ng/ml to about 3.0 ng/ml of serum.
44. The method of claim 3, wherein said sample is obtained at day 20 post-insemination, and the elevated levels of UCRP and progesterone are at least 2 ng/ml and about 2 to 5 ng/ml respectively.
45. The method of claim 1, further comprising a positive control sample from a pregnant bovine animal.
46. The method of claim 1, further comprising a negative control sample from a non-pregnant bovine animal.
47. The method of claim 1, further comprising measuring UCRP and progesterone levels from a second sample from bovine animal at a second point in time.
48. A method of making a breeding decision for an animal comprising:
- (a) obtaining a sample from said animal, wherein said animal is suspected of being pregnant;
 - (b) measuring the level of UCRP in said sample; and
 - (c) measuring the level of progesterone in said sample, wherein:
 - (i) elevated levels of UCRP and progesterone indicate that said animal is pregnant, and no further steps need be taken;
 - (ii) non-elevated levels of UCRP and progesterone indicate that said animal is not pregnant, and should be injected with gonadotropin-releasing hormone

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- (GnRH), and about seven days later, injected with prostaglandin $F_{2\alpha}$ (PGF), followed by re-insemination;
- (iii) elevated levels of UCRP and non-elevated levels of progesterone indicate that said bovine animal is not pregnant due to early embryo death and should be injected with GnRH, and about seven days later, injected with PGF, followed by re-insemination; or
- (iv) non-elevated levels of UCRP and elevated levels of progesterone indicate that said bovine animal is not pregnant, and should be injected with PGF, followed by re-insemination.
- 5
- 10 49. The method of claim 48, further comprising in step (ii), (iii) or (iv), about 48 hours after PGF injection and before re-insemination, administering a second injection of GnRH.
50. The method of claim 48, further comprising, prior to step (a), inseminating said bovine animal.
51. The method of claim 48, wherein said PGF injection is administered at day 20 post-insemination and wherein said re-insemination is carried out at day 28 post-insemination.
- 15 52. The method of claim 48 wherein said PGF injection is administered at day 26 post-insemination and wherein re-insemination is carried out at day 28 post insemination.
53. A method for the detection of pregnancy in an animal comprising:
- (a) obtaining a sample from said animal; and
- 20 (b) immunologically measuring the level of an ubiquitin cross-reactive protein (UCRP) in said sample by means comprising the use of an antibody raised to full-length UCRP;
- wherein elevated levels of UCRP indicate that said bovine animal is pregnant.
54. The method of claim 53, wherein said sample is saliva, serum, blood, milk or urine.
- 25 55. The method of claim 54, wherein said sample is serum.
56. The method of claim 53, wherein said sample is obtained from said animal at day 16 to 28 post-insemination.

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57. The method of claim 53 wherein the animal is a ruminant.

58. The method of claim 57 wherein the ruminant is a beef or dairy cow.

59. A method of making a breeding decision for an animal comprising:

(a) obtaining a sample from said animal, wherein said animal is suspected of being pregnant; and

(b) immunologically measuring the level of UCRP in said sample by means comprising the use of an antibody raised to full-length UCRP ;

wherein:

(i) elevated levels of UCRP indicate that said animal is pregnant, and no further steps need be taken;

(ii) non-elevated levels of UCRP indicate that said animal is not pregnant, and should be either injected with gonadotropin-releasing hormone (GnRH), and about seven days later, injected with prostaglandin $F_{2\alpha}$ (PGF), followed by re-insemination or should be injected with PGF, followed by re-insemination.

1/1

	ISG 17	Progesterone		ISG 17	Progesterone	Cow
Test						
Control						
Test						
Control						
Test						
Control						
Test						
Control						

			+	+		Pregnant
			+	-		Open
			-	+		Open
			-	-		Open

FIGURE 1

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<120> Method and Means for Early Detection of Pregnancy in Animals by Combination Testing

<130> 11916.0056.00PC00 (MOPV056P)

<150> U.S. 60/435,540

<151> 2002-12-19

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			20					25					30		

Ala Gln Lys Ile Asn Val Pro Ala Phe Gln Gln Arg Leu Ala His Leu
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Asp Ser Arg Glu Val Leu Gln Glu Gly Val Pro Leu Val Leu Gln Gly
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 Leu Arg Ala Gly Ser Thr Val Leu Leu Val Val Gln Asn Cys Ile Ser
 65 70 75 80
 Ile Leu Val Arg Asn Asp Lys Gly Arg Ser Ser Pro Tyr Glu Val Gln
 85 90 95
 Leu Lys Gln Thr Val Ala Glu Leu Lys Gln Gln Val Cys Gln Lys Glu
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 Arg Val Gln Ala Asp Gln Phe Trp Leu Ser Phe Glu Gly Arg Pro Met
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Met Gly Gly Asp Leu Thr Val Lys Met Leu Gly Gly Gln Glu Ile Leu
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 20 25 30
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 35 40 45
 Asp Ser Arg Glu Val Leu Gln Glu Gly Val Pro Leu Val Leu Gln Gly
 50 55 60
 Leu Arg Ala Gly Ser Thr Val Leu Leu Val Val Gln Asn Cys Ile Ser
 65 70 75 80
 Ile Leu Val Arg Asn Asp Lys Gly Arg Ser Ser Pro Tyr Glu Val Gln
 85 90 95
 Leu Lys Gln Thr Val Ala Glu Leu Lys Gln Gln Val Cys Gln Lys Glu
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 Arg Val Gln Ala Asp Gln Phe Trp Leu Ser Phe Glu Gly Arg Pro Met
 115 120 125
 Asp Asp Glu His Pro Leu Glu Glu Tyr Gly Leu Met Lys Gly Cys Thr
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 Val Phe Met Asn Leu Arg Leu Arg Gly Gly
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